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# Isolation of *Trypanosoma cruzi* DNA in 4,000-Year-Old Mummified Human Tissue From Northern Chile

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*ABSTRACT* A segment of DNA unique to the kinetoplast of *Trypanosoma cruzi* was isolated from spontaneously mummified human remains from the coastal area of northern Chile at sites dated from 2000 BC to about AD 1400. Following rehydration of the desiccated human tissue samples of heart, esophagus, or colon, the samples were extracted and primers employed to bind to a 330 bp kinetoplast minicircle DNA sequence present in *T. cruzi*. This segment was then amplified using the polymerase chain reaction (PCR), and the target segment was visualized by gel electrophoresis. This method enables the identification of Chagas' disease in an ancient body in the absence of recognizable anatomic pathological changes. Am J Phys Anthropol 108:401–407, 1999. 

We report the successful identification of the presence of *Trypanosoma cruzi* in up to 4,000-year-old human remains from South America using molecular biology methods. This represents a pilot study of a much more extensive investigation designed to identify the antiquity, demography, and especially the paleoepidemiology of Chagas' disease (American trypanosomiasis) in the New World.

## EPIDEMIOLOGY AND TRANSMISSION OF CHAGAS' DISEASE

Compared with tropical diseases such as malaria and leishmaniasis, Chagas' disease is considered to cause the greatest socioeconomic burden in tropical America today (Schmunis, 1994). It was first identified by the Brazilian scientist Carlos Chagas (1909). It is a tropical disease caused by the protozoan parasite *Trypanosoma cruzi* and is transmitted principally through the feces of reduviid (kissing or assassin bug; "vinchuca") bugs belonging to the subfamily *Triatominae*. However, other forms of transmission include organ transplantation, blood transfusion, and oral and congenital routes (Aufderheide, 1995). The vectorial transmission of *T. cruzi* is restricted to the New World. The geographic distribution of triatomine species extends from the Neotropical to the Neoarctic regions. They can be found from Utah in the United States to

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Patagonia in South America (World Health Organization, 1991). The most common triatomine in the southern part of South America is *Triatoma infestans*, usually found in rural dwellings inside the cracks of walls and roofs. Several programs are active in the control of *T. cruzi* in tropical America (Guhl and Schofield, 1996).

The traditional epidemiological pattern of *Trypanosoma cruzi* reveals that primitive transmission was restricted to specific cycles in tropical forest environments, where triatomines would feed on small mammals in broad areas of the South American continent, without humans intervening in the natural cycle (Barretto, 1979). The same situation persists today in wild areas configuring a wild enzootic epidemiological character. The presence of *T. cruzi* does not seem to affect triatomines significantly nor the mammals which have been naturally infected. This would suggest a balance between species as a result of long periods of adaptation. Although in general the *Hemiptera* represent an ancient order, with fossilized remains dating from the Permian (nearly 232–280 mya), it is possible that the evolution of triatomines and other haematophagous groups corresponded to a series of later events starting at different times and from diverse ancestral forms. The *Hemiptera* comprise the largest order of exopterygote insects, with over 80,000 widely distributed species in all tropical and temperate areas (Schofield, 1994). Ancestral predatory habits amongst the triatomines can be inferred by the fact that some species occupy a relatively wide spectrum of ecotopes and are able to exploit different species of hosts. Others, on the contrary, occupy restricted habitats and hosts (Schofield and Matthews, 1985).

Human Chagas' disease is a purely accidental occurrence. As humans came into contact with the natural foci of infection and caused different degrees of ecological transformations, triatomines were forced to occupy their dwellings. Thus began a process of adaptation to human habitations through which the insects had direct access to abundant food as well as protection from climatic changes and predators. For example, *Triatoma infestans* is the main vector of *T. cruzi* in the southernmost countries of South America and is considered an almost exclusively domiciliary species.

Clinically the disease is characterized by an initial stage (usually in children) of parasitemia secondary to wound contamination by parasite-laden triatomine feces following the insect bite. Multiple organs are infected, but acute myocarditis or occasionally encephalitis are responsible for up to 10% fatalities in this stage. Clinical recovery is then often followed by a prolonged (up to decades) interval with no or occasional symptomatic parasitemia episodes. Late complications include dilated cardiomyopathy as well as segmental areas of destruction of myenteric plexus ganglion cells with resulting peristaltic paralysis and dilatation of affected gut areas (most commonly megacolon and megaesophagus). Prevalence estimates suggest that up to 18 million Latin Americans are infected with incurable American trypanosomiasis.

## CHAGAS' DISEASE IN MUMMIFIED REMAINS

Some reports have suggested that the adaptation of triatomines to human habitats could have occurred as early as the sixth century AD in northern Argentina (Carpintero and Viana, 1980). A few authors have dedicated their efforts to the identification of *T. cruzi* in mummified human tissues through the application of immunochemical and electron microscope techniques. Fornaciari et al. (1992) reported the infection of human tissue by *T. cruzi* in a Peruvian Inca mummy using these methods. Furthermore, gross morphopathological evidence for the presence of prehistoric Chagas' disease diagnosed from anatomic pathological changes (megasyndromes) in ancient Chilean mummies has been presented by Rothhammer et al. (1985) with radiocarbon dates ranging between 470 BC to AD 600. Associated cultural contexts indicated the presence of mud brick dwellings, these being a common habitat for triatomines.

The detection of organic compounds in ancient remains has opened a new research area with many implications, the most important being the extraction of ancient DNA (aDNA) from fossils, biological sources, bone,

and mummified human bodies. The high conservation of sequences of kinetoplast DNA (kDNA) minicircles in *T. cruzi* and *T. rangeli* (Vallejo et al., 1994) allows the detection of *T. cruzi* in modern tissues by the amplification of a 330 bp fragment and *T. rangeli* by 760 bp (Macedo et al., 1995) using primers S35 and S36. Conventional diagnostic methods for detecting parasitaemia in chronic *T. cruzi* infection in live patients present very low sensitivity. The polymerase chain reaction (PCR) used to detect parasite kinetoplast DNA (kDNA) in modern samples has been shown to be virtually 100% specific and sensitive in chronically infected individuals (Centurion-Lara et al., 1994). Moreover, the sensitivity for *T. cruzi* kDNA detection was estimated as 0.1% of the content of mitochondrial DNA (mtDNA) from a single parasite cell (Sturm et al., 1989).

Bastos et al. (1996) have demonstrated that this technique is also capable of recovering kinetoplast DNA from modern trypanosome-infected tissues that have been desiccated and subsequently rehydrated, but we are not aware of reports in which the PCR approach has been successful in isolation of trypanosome DNA from millennia-old ancient tissues.

## PURPOSE OF THIS STUDY

The question we asked in this investigation was whether current molecular methods can extract, amplify, and identify a 330 bp segment of *kDNA* unique to *Trypanosoma cruzi* from mummified human tissue even if the mummified tissue is as much as 4000 years old and if the tissue reveals no morphological changes sometimes produced by *T. cruzi*. Our results of aDNA analysis using the primers reported here confirm the presence of Chagas' disease in early South American mummies during prehistoric times in northern Chile.

## MATERIALS AND METHODS

### Mummified human visceral tissue and bone samples

The specimens from mummified human remains were obtained by anatomic dissection of the spontaneously desiccated human remains from the coastal areas of the Atacama desert of northern Chile between about 18° and 20° south latitude from Chiribaya Alta sites near the city of Ilo, Peru (Cha), the Alto Ramirez site near Pisagua (Psg) in Chile, and sites near Arica, Chile: Azapa (Az), Camarones (Cam), Morro (Mo), and Sin Referencia (Sr). These bodies had been interred without human effort to preserve soft tissues, but the temperature, aridity, and soil characteristics combined to dehydrate the body tissues spontaneously before decay processes destroyed them. The Chinchorro people (Mo) initiated coastal colonization of river mouths of this area about 7500 BC and practiced a maritime economy there until about 1500 BC. Although they mummified some of their bodies by anthropogenic methods (Arriaza, 1995), the samples for our study (Mo1–6, about 2000 BC) were obtained from spontaneously mummified remains. Members of the highland cultures in the region of Lake Titicaca settled in the coastal areas about 1000 BC, introducing agropastoral subsistence practices (specimens Psg). Following subsequent highland colonization, the coastal-valley cultures (represented by specimens Az-75, Cha, Sr, and finally Cam) expanded their agricultural technology until the sixteenth century Spanish conquest (specimens Az-142). These specimens were acquired by dissections in communities near the site and kept in waterproof and airtight containers at laboratory room temperature until analysis. Specimens For-1 are from a Peruvian Inca (circa AD 1400) adult female mummy in whose tissues *T. cruzi* amastigotes had been identified previously by electron microscopy and immunostaining (Fornaciari et al., 1992); these specimens were employed in this study as a form of positive control sample. Figure 1 demonstrates a dilated esophagus in such human remains. Similarly, specimens (Eg) from a spontaneously mummified ancient Egyptian body (about 200 BC) from the Dakhleh Oasis were included as a negative control since Chagas' disease is limited to the New World.

Age estimates for adults were primarily determined by symphysis pubis criteria as described in Ubelaker (1989) before 1990 and by those of Suchey et al. (1988) after that date. Retention of external genitalia and/or breasts in many mummies permitted


Fig. 1. Az-75, T-43: 35–40-year-old female from a valley site in extreme northern Chile about AD 1300. The opened lower esophagus (A) measured 8.0 cm in circumference in its desiccated condition, about a fourfold increase above normal. A trypanosome target kinetoplast DNA was isolated from a sample of this esophagus.

ready identification of sex, and conventional pelvic and cranial criteria were employed for the remainder. Visceral and other soft tissue preservation varied enormously and was not necessarily related to the time interval between death and excavation. Thus, not all organs were available for sampling in all bodies. The structures sampled in this study were those commonly affected in modern patients with Chagas' disease (heart, stomach, esophagus, and colon) when these were present. Marrow-containing bone (rib, sternum) were included as a surrogate sample for peripheral blood. More details about these mummies can be found in Aufderheide (1996), Aufderheide et al. (1994), and Cartmell et al. (1991).

### DNA extraction from mummified tissues

Approximately 3 × 3 mm pieces of mummy tissue sample were rehydrated in 0.5% trisodium phosphate solution at room temperature for 48 h. These samples were centrifuged for 15 min at 1,500*g*. The supernatant was discarded, and 2.5 ml TENS was added (TrisHCl 50 mM, EDTA 50 mM, NaCl 100 mM, SDS 0.5%) together with 10 µl proteinase K (stock 10 mg/ml) two times a day for 3 days, keeping the samples at 37°C for the digestion of proteins.

To 200 µl of digested sample, 200 µl phenol was added, placed in tube number 1, and then gently mixed for 1 min. After centrifugation for 5 min in a microfuge, the supernatant was collected in tube 2, and 100 µl phenol and 100 µl chloroform were added. After gentle shaking for 1 min, the centrifugation was repeated and the supernatant collected in tube 3. Then 150 µl of $H_2O$ was added in tube 2 in order to obtain a greater amount of DNA by repeating the previous procedure. The supernatants were collected in tube 3, and the same volume of chloroform was added to the aqueous phase obtained. DNA was precipitated by adding 10% of the measured volume of sodium acetate and twice the volume of absolute ethanol at −20°C and precipitated in ice for 15 min. The supernatant was discarded after 15 min of centrifugation in a microfuge. The pellet was dried 10 min at 50°C and resuspended in 30 µl $H_2O$ (M.L. Gomes, personal communication).

### DNA extraction from blood samples

Blood samples to which cultivated *T. cruzi* had been added and trypanosome-free blood were used as controls and were extracted with guanidine EDTA. An equal volume of guanidine 6 M/EDTA 0.2 M, pH 8, was added. The mixture was left at room temperature for 7 days. In order to release the kDNA minicircles, we boiled 200 µl aliquots for 15 min (Britto et al., 1993) and kept them at room temperature for another 7 days. DNA was extracted following the procedure mentioned above.

### Amplification of *T. cruzi* DNA

For each polymerase chain reaction (PCR) assay, a DNA thermal cycler was used (PTC-100; MJ Research Inc., Watertown, MA). The PCR was done using primers S35 (5′-AAATAATGTACGGG-KGAGATGCATGA-3′) and S36 (5′-GGTTCGATTGGGGTTGGT-GTAATATA-3′) (Avila et al., 1991; Britto et al. 1993) which amplify a segment of 330 bp length from the variable regions of the minicircles of kDNA in *T. cruzi*.

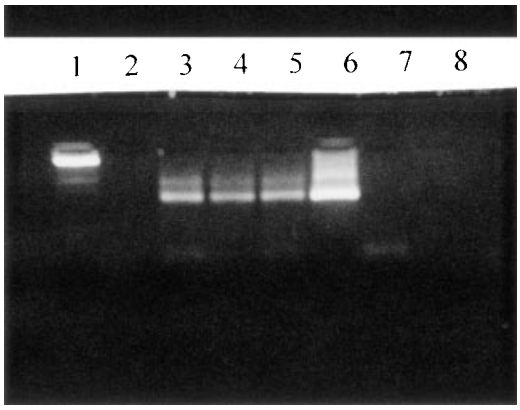


Fig. 2. Polymerase chain reaction products. Aliquots (10 µl) were loaded and separated by electrophoresis. Lane 1: Molecular weight markers lambda HindIII. Lane 2: Negative control, mummy Eg-1 muscle tissue. Lane 3: Positive control, mummy For-1 heart tissue. Lane 4: Mummy Az-75, T-43, esophagus tissue. Lane 5: Mummy Az-75, T-133, C-1, heart tissue. Lane 6: *T. cruzi* stock tulahuen from culture. Lane 7: Trypanosome-free human blood. Lane 8: Control reaction, $H_2O$.

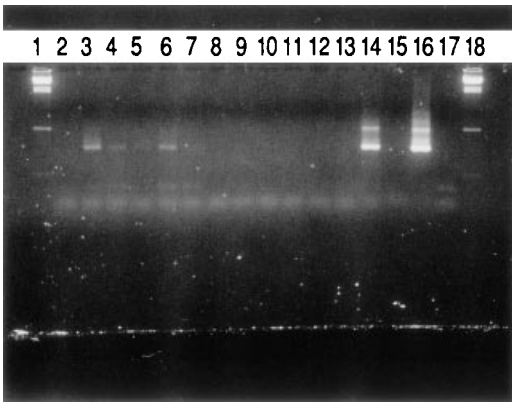


Fig. 3. Polymerase chain reaction products. Aliquots (10 µl) were loaded and separated by gel electrophoresis. Lane 1: Molecular weight markers lambda HindIII. Lane 2: Negative control mummy Eg-1 muscle tissue. Lane 3: Positive control, mummy For-1 heart tissue. Lane 4: Mummy Mo1–6, T-39, esophagus tissue. Lane 5: Mummy Cam-9, T-5, heart tissue. Lane 6: Mummy Psg-7, T-726B, heart tissue. Lane 7. Mummy Cha-2, Aut-5, colon tissue. Lane 8: Mummy Cha-1, Aut-80, heart tissue. Lane 9: Mummy Cam-9, T-16, sternum tissue. Lane 10: Mummy Az-142, C-5, heart tissue. Lane 11: Mummy Cha-2, Aut-51, heart tissue. Lanes 12,13: Mummy Az-141, T-52, heart and colon tissues, respectively. Lane 14. Human blood, *T. cruzi* added. Lane 15: Trypanosome-free human blood. Lane 16: *T. cruzi* stock tulahuen from culture. Lane 17: Control reaction, $H_2O$. Lane 18: Molecular weight markers lambda HindIII.

The following protocol was used for a 20 µl reaction: buffer 10X (Promega, Madison, WI), $MgCl_2$ 2.5 mM (Promega), KCl (50 mM), dNTPs (200 µM) S35 and S36 10 picomoles each, Taq 1 unit (Promega). Then 2 µl of the extracted DNA was added for each 20 µl of reaction. The thermal profile used was 35 cycles at 94°C for 1 min, at 60°C for 1 min, and 72°C for 1 min, an initial denaturation step of 5 min at 94°C, and a final extension at 72°C over a period of 5 min. An aliquot of 10 µl was electrophoresed in 2% agarose gels for visualization of the amplified DNA. In this study, positive identification of *T. cruzi* kDNA was defined as the isolation of a 330 bp band on the electrophoresis gel.

DNA extraction, PCR reagents mix, and electrophoresis were done in separate rooms. All materials were previously irradiated with ultraviolet (UV) light. Other common measures such as the use of aerosol-resistant tips were also employed.

## RESULTS AND DISCUSSION

Figures 2 and 3 display characteristic results of our study. Table 1 lists the results of amplification studies on all mummy samples tested.

As expected, the heart, esophagus, and colon samples from For-1 specimens (positive control) were positive, while the Eg-1 specimens (Egyptian mummy tissues incorporated into the study as a negative control) consistently failed to amplify the target DNA segment.

Of the various tissues from 27 mummies tested, those from nine mummies demonstrated a positive reaction in at least one of the examined organs. This must not be interpreted as having any prevalence significance because dilatation of the heart, esophagus, or colon was deliberately included in the selection of mummies for this pilot study. Indeed, only four mummies in whom none of these structures were dilated were tested, and at least one specimen from each of three of these four demonstrated amplified trypanosome DNA. Five of the nine dilated hearts were positive, as were all four esophagus samples, only two of which were dilated. However, the specimen from only one of the mummies demonstrating a dilated colon segment amplified the target DNA segment,

*TABLE 1. Characteristics of tested mummies*[1]

| MT number | Mummy field number | Positive test[2] | Age (years) | Sex | Individual tissue tested | | | | | Time period | Other findings |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Heart | Esophagus | Stomach | Colon | Bone | | |
| 1 | Mol-6, T-18 | O | 35–45 | M | N O | | | | | 2000 BC | |
| 2 | Mol-6, T-33 | O | 14–16 | M | N O | | | | | 2000 BC | Large number of coprolites |
| 3 | Mol-6, T-39 | + | 12–14 | F | | N+ | | N O | | 2000 BC | Ileum: + |
| 4 | Psg-7, T-721 | O | 40–45 | F | N O | | | | R O | 1000 BC | |
| 5 | Psg-7, T-723 | + | 25–35 | M | N + | | | N O | | 1000 BC | Muscle: O |
| 6 | Psg-7, T-726 | + | 5–7 | I | D + | | | | R O | 1000 BC | Pneumonia, acute, LUL |
| 7 | Eg-1 | O | 12–17 | N | | | | | | 200 BC | Liver: O; lung: O; muscle: O |
| 8 | Az-141, T-36 | O | 16–19 | M | D O | | | | | AD 800 | Secondary hyperparathyroidism |
| 9 | Az-141, T-38 | O | 25–35 | M | N O | | | | | AD 800 | Tuberculosis |
| 10 | Az-141, T-52 | O | 35–45 | M | N O | | | D O | | AD 800 | |
| 11 | Chi-95, T-4 | O | 12–16 | M | D O | | | | | AD 1200 | |
| 12 | Cha-2, Aut-5 | O | 20–23 | F | | | | D O | | AD 1200 | |
| 13 | Cha-1, Aut-6 | O | 18–20 | F | | | | N O | | AD 1200 | |
| 14 | Cha-2, Aut-51 | O | 25–28 | F | D O | | | | | AD 1200 | |
| 15 | Cha-1, Aut-80 | O | 35–45 | M | D O | | | | | AD 1200 | Pneumonia, acute, LUL |
| 16 | Plm-6, T-19 | O | 30–40 | F | N O | | | | | AD 1250 | |
| 17 | Az-75, T-43 | + | 35–40 | F | | D+ | D O | | | AD 1350 | |
| 18 | Az-75, T-85, C-1 | O | 3–5 | F | N O | | | | | AD 1350 | |
| 19 | Az-75, T-103 | + | 25–30 | F | D + | | | | | AD 1350 | |
| 20 | Az-75, T-133, C-1 | + | 45–55 | M | D + | N + | | | | AD 1350 | Pneumonia, acute, RUL |
| 21 | Sr-1, T-3 | + | 13–15 | F | D + | | | | | AD 1350 | Pott's disease |
| 22 | Cam-9, T-1 | O | 30–40 | M | N O | | | | | AD 1400 | |
| 23 | Cam-9, T-5 | + | .5–1.5 | M | N + | | | | | AD 1400 | |
| 24 | Cam-9, Aut-16 | O | 40–45 | F | | | | | S O | AD 1400 | |
| 25 | For-1 | + | 18–21 | F | D + | D + | | D + | | AD 1400 | Ileum: O; lung: O; rectum: O |
| 26 | Az-142, T-5 | O | 50–60 | M | N O | | | | | AD 1800 | Anthracosilicate pneumoconiosis |
| 27 | Lim-1, T-1 | O | 20–30 | M | N O | | | | | AD 1800 | |

[1] D, organ was dilated; MT, mummy test number; N, organ was not dilated; O, no amplification of DNA target occurred; R, rib; S, sternum; +, amplification of DNA target occurred.
[2] Positive test, at least one sample amplified DNA target.

and that one was found to contain an enormous quantity of coprolites (dried feces), a common feature in persons with Chagas' disease because of the arrest of intestinal motility secondary to intestinal wall nerve cell destruction by the parasite. This suggests the colon may become dilated in mummified remains by processes other than trypanosome infections, probably by postmortem growth of gas-forming colon bacteria. Tissues from two of three tested children (ages 1 and 6 years) reacted positively. These may have died during the acute stage during dissemination of the parasite by the blood. The oldest individual with positive reactions was 50 years old; those in the later adult years were probably in the chronic phase of the infection.

These results indicate that the procedure employed is capable of extracting and amplifying a segment of kinetoplast DNA unique to *Trypanosoma cruzi* present in the spontaneously desiccated tissues of ancient human remains at least as old as 4,000 years in individuals whose age at death ranged from 1–50 years. To date, paleopathology has depended largely on morphological diagnostic criteria. This type of methodological approach provides the opportunity to identify a specific infection in an ancient human body that bears no detectable gross anatomic lesion of the infection, providing the potential for a substantial expansion of the range of diseases that can be detected in ancient remains.

We are aware of a recent international initiative among several South American countries to study Chagas' disease as a common public health problem. Hence, we felt that publication of the positive results

we obtained in this initial phase of our project may have relevance to other laboratories dealing with this disease.

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